Grants # 209 &
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The Bacterial Oxidation of Nicotine

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(Received for publication, August 18, 1952)

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 234, No. 1, January, 1959
Made in United States of America

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II. THE ISOLATION OF THE FIRST OXIDATIVE PRODUCT AND ITS IDENTIFICATION AS (1)-6-HYDROXYNICOTINE*

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(Received for publication, August 18, 1958)

It has been previously shown (1) that cell-free extracts from a soil bacterium, P-34, oxidize nicotine with the consumption of 0.5 μmole of oxygen per μmole of nicotine, producing a compound which possesses a characteristic ultraviolet absorption spectrum. Preliminary data suggested that this compound was either a 2- or 6-pyridone derivative of nicotine. This paper presents evidence which indicates that this compound is the first oxidative product of nicotine degradation and is indeed (1)-6-hydroxynicotine (6-OHN).[1]

EXPERIMENTAL

The conditions of growth and the preparation and fractionation of cell-free extracts have been described previously (1).

2-OHN was synthesized from nicotine according to the methods of Chicababin and Kirssanow (2) and Wada (3). Samples of 2-OHN and 6-OHN were obtained as gifts through the generosity of Dr. E. Wada, Central Research Institute, Japan Monopoly Corporation, Tokyo, Japan.

Infrared spectra were determined with a Perkin-Elmer model 137 Infracord double beam spectrophotometer. The sample was made up in potassium bromide by mixing 1 part of sample with 100 parts of anhydrous potassium bromide. The mixture was agitated for 2 minutes in a Wig-L-Bug amalgamator,[2] and the resulting powder was compressed at 18,000 pounds/sq. in. in a vacuum.

Ultraviolet spectrophotometry, paper chromatography, and manometry were carried out as previously described (1). Melting point determinations were made in sealed capillary tubes and were uncorrected. Optical rotations were determined in a Rudolph High Precision Polarimeter, model 80.

RESULTS

Isolation of First Oxidative Product from Enzymatic Reaction Mixtures—2000 μmoles of nicotine (324 mg.), 10 μmoles of methylene blue, 490 μmoles of potassium phosphate buffer, pH 7, and the 20 to 40 fraction were incubated in an Erlenmeyer flask in a total volume of 20 ml. The flask, attached to a mercury manometer, was placed on a rotary shaker and incubated at 30°.

When oxygen consumption approached 0.5 μmole of oxygen per μmole of nicotine, 2 volumes of 0.1 N HCl were added to the reaction mixture and the precipitated protein was removed by centrifugation. The acidified solution was treated with an excess of Dowex 50 in the acid form until the supernatant solution failed to give a precipitate with STA. The resin was centrifuged, washed with 0.1 N HCl, and suspended in 0.1 M KCl. The resin was removed by centrifugation, the eluate was saved, and the resin was suspended in fresh 0.1 M KCl and again centrifuged. The combined eluates were evaporated to dryness at reduced pressure to yield a yellowish residue. This was exhaustively extracted with several portions of boiling Skellysolve B and the solvent fractions were combined and partially evaporated. Crystallization was permitted to occur at room temperature yielding an amorphous solid having a yellowish tinge. A second crystallization yielded a material which melted at 109–119°. After treatment with Norit A, recrystallization from boiling Skellysolve B yielded a white crystalline material which melted at 120–122° (yield, 144 mg.).

Isolation of First Product from Growth Medium—During growth of P-34 in a nicotine-yeast extract-mineral salts medium, a product accumulated whose absorption spectrum was similar to the substance produced by the 20 to 40 fraction. Preliminary studies indicated that the accumulation was maximum after 36 hours of growth and that it was accompanied by the formation of a greenish fluorescent pigment (4).

In order to isolate the product, 28 l. of the growth medium were distributed in 7-l. lots and incubated as previously described. After 36 hours of growth, the cells were collected in a Sharples centrifuge and the greenish supernatant fluid was acidified to pH 2 with concentrated HCl and treated with STA to precipitate the product. An aqueous neutral solution of the product was obtained from the STA salt by following the procedure of Frankenburg *et al.* (5). This solution was evaporated to dryness and treated with Skellysolve B as previously described. After 3 recrystallizations from Skellysolve B, 8 gm. of a white crystalline material were obtained (m.p. 121–121.5°).

Metabolic Behavior of Product—Nicotine-grown resting cells oxidized the isolated product and nicotine at the same rate (Fig. 1). At the cessation of oxidation the consumption of oxygen in

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[1] The abbreviations used are: 2-OHN, 2-hydroxynicotine; 6-OHN, 6-hydroxynicotine; STA, silicotungstic acid.

[2] Manufactured by Crescent Dental Manufacturing Company, Chicago, Illinois.

the case of the product was 0.5 μmole of oxygen per μmole of substrate less than in the case of nicotine.[3]

Crude extracts oxidized nicotine and the product at the same initial rate (Fig. 2). In the case of nicotine oxidation, the rate of oxygen uptake changed after the consumption of 1 μmole of oxygen per μmole of nicotine. The rate of product oxidation changed after the consumption of 0.5 μmole of oxygen per μmole of substrate. After these changes in rates, the rates of oxygen consumption for nicotine and product oxidation were essentially the same. The product was not oxidized by the 20 to 40 fraction which oxidized nicotine only to the 0.5 μmole level (1). With the product as substrate, the 40 to 60 fraction (which oxidizes nicotine only to the 1 μmole level) and the crude extract consumed the same amount of oxygen at the cessation of oxidation and at the first change in rate respectively (Table I).

The stoichiometry of oxygen consumption by resting cells as well as the stoichiometry and rate of oxygen consumption by various cell-free extracts indicate that the product is an intermediate in nicotine degradation and not a product of a side reaction.

Properties of Product—The products isolated from enzymatic reaction mixtures and growth medium were judged to be identical by the following criteria: identity of ultraviolet absorption spectra; similarity of melting points; no significant depression on mixed melting point determinations; and identical behavior during chromatography (Table II).

The product did not give rise to a chromogenic compound when treated with cyanogen bromide and β-naphthylamine according to the method of McCormick and Smith (6). The presence of a hydroxyl group was indicated by the formation of a deep burgundy color with ceric nitrate (7). Upon standing, the color faded. That this functional group was a pyridone seemed probable from the nature of the ferric chloride reaction. The product reacted with ferric chloride under acid conditions (in 0.2 N HCl), but not under neutral conditions, giving rise to an orange-red color, a behavior typical of pyridones (8).

An elemental analysis gave[4] the following results:

$C_{10}H_{14}N_2O$

Calculated: C 67.42, H 7.86, N 15.73

Found: C 67.46, H 8.00, N 15.62

The product was optically active, exhibiting an $(\alpha)_D$ of −54.8° in aqueous solutions.

Although the melting point of the product was similar to the reported melting point of 2-OHN (9), comparison of the product with synthetic samples of 2-OHN indicated that the substances were not identical. The ultraviolet absorption spectra of 2-OHN and the product differed significantly. The absorption maxima of 2-OHN were located at 228 and 303 mμ while those of the product were located at 232 and 295 mμ (Fig. 3). Furthermore, the $A_{232/295}$ for 2-OHN was considerably lower than that for the biological product. 2-OHN failed to give rise to chromogenic compounds when treated with either ceric nitrate or acidic ferric chloride. The picrate derivative of 2-OHN melted some 30° higher than did the corresponding derivative of the biological product (Table II). 2-OHN did not prove to be metabolically active. Crude extracts which oxidized nicotine beyond the oxidation level of the biological product did not oxidize 2-OHN. Finally, and most significantly, the infrared spectra of the two compounds were markedly different. The absorption spectrum of 2-OHN exhibited a pronounced band at approximately 13 μ whereas the absorption spectrum of the biological product lacked this feature (Fig. 4).

On the other hand, several properties exhibited by synthetic 6-OHN suggested that it might be identical to the metabolic product. Its ultraviolet absorption spectrum was essentially identical to that of the metabolic product (Fig. 3). The $A_{232/295}$ of both compounds was in good agreement. Authentic 6-OHN reacted with ceric nitrate and ferric chloride in a manner identical

[3] The product was assumed to be a hydroxynicotine, molecular weight 178, for this calculation.

[4] The analysis was performed by Dr. A. Elek, Elek Micro Analytical Laboratories, Los Angeles, California.

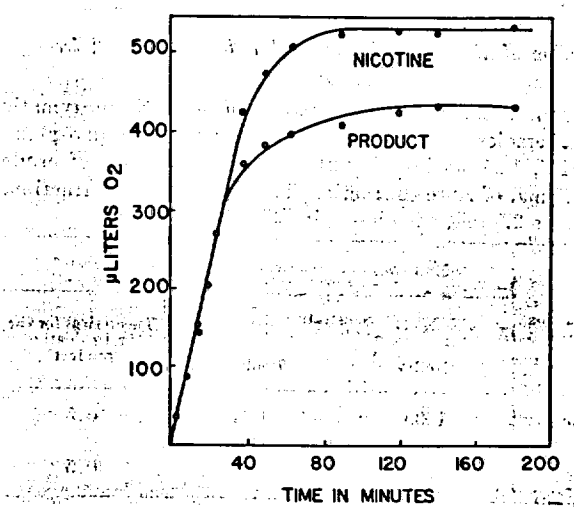

FIG. 1. The oxidation of nicotine and the first oxidative product by nicotine-grown resting cells. Experimental conditions: 3.8 μmoles of nicotine, 3.5 μmoles of product, 70 μmoles of potassium phosphate buffer, pH 7, 0.25 ml. of a resting cell suspension. Total volume 2.0 ml., gas phase air, 30°.

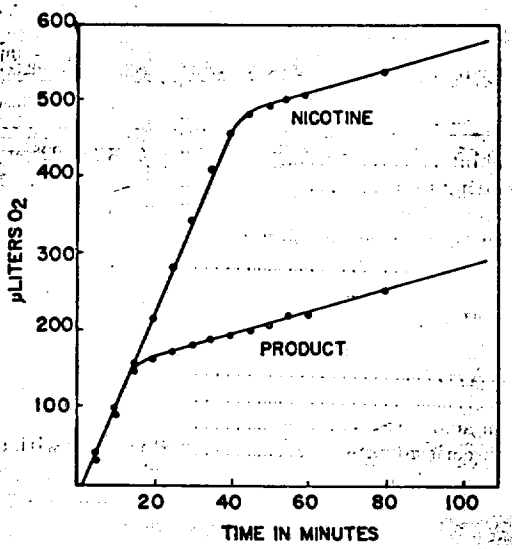

FIG. 2. The oxidation of nicotine and the first oxidative product by a crude extract. Experimental conditions: 20 μmoles of nicotine, 15 μmoles product, 102 μmoles of potassium phosphate buffer, pH 7, 1.25 μmoles of methylene blue, and 17.5 mg. of crude extract. Total volume, 2.0 ml., gas phase air, 30°.

TABLE I

Oxidation of nicotine and first product by various cell-free extracts

Experimental conditions: 19 μmoles of 6-OHN (enzymatic product), 20 μmoles of nicotine, 102 μmoles of potassium phosphate buffer, pH 7, 1.25 μmoles of methylene blue, 17.5 mg. of crude extract, 7.5 mg. of 20 to 40 fraction, 14.5 mg. of 40 to 60 fraction. Total volume 2.0 ml., gas phase air, 30°.

Enzyme fraction	μMoles of oxygen consumed per μmole of substrate*		
	Substrate		Theoretical for the first oxidative product
	Nicotine	Product	
Crude	1.09	0.41	0.5
20 to 40	0.48	0	0
40 to 60	1.05	0.43	0.5

* For the crude extract, the oxygen consumption reported is to the first change of rate; all other values represent oxygen consumption at the cessation of oxidation.

III). This would indicate that crude extracts contain an enzyme capable of racemizing 6-OHN.

At the cessation of oxidation of the biological product by the 40 to 60 fraction, the absorption spectrum of the reaction mixture changed. The absorption maximum at 232 mμ disappeared and the maximum at 295 mμ shifted to 290 mμ. Thus the change in absorption was similar to that observed in reaction mixtures in which crude extracts had oxidized nicotine with the consumption of 1 μmole of oxygen per μmole of nicotine prior to the change in oxidative rate (1).

DISCUSSION

The characterization of the first oxidative product of nicotine degradation by strain P-34 as (1)-6-OHN confirms, at least in part, the earlier report of Frankenburg and Vaitekunas (10) who isolated 6-OHN during the fermentation of nicotine by a tobacco seed infusion. The product isolated by these workers was apparently a racemic mixture as judged by the reported melting point of their compound and its failure to depress the melting point of a synthetic sample of 6-OHN. Whether racemization occurred as a consequence of their isolation procedure or was due to enzymatic action is difficult to assess because of the complexity of the environmental conditions which were employed as well as the failure of these workers to describe their isolation procedure. In view of the isolation of 6-OHN and other compounds from fermentation mixtures, these authors postulated 3 pathways for the bacterial degradation of nicotine: the "pyridine pathway," in which 6-OHN is the first oxidative product; and the "pyrrolidine pathway," which in reality consisted of two pathways which diverged after the formation of γ-keto-γ-(3-pyridyl) butyric acid, in which pseudo-oxynicotine was proposed as the first product.

Wada (3), who investigated the oxidation of nicotine by a group of soil bacteria, isolated and identified a series of compounds from the spent growth medium. He proposed that nicotine degradation is initiated at the pyrrolidine ring to yield *N*-methylmyosmine as its hydrated species, pseudo-oxynicotine. Some of the soil isolates were also capable of degrading nor-

TABLE II

Properties of biological product and synthetic 2- and 6-OHN

Property	Biological products		Synthetic products	
	Growth medium	Enzymatic reaction	2-OHN	6-OHN
Melting point	120–122°	121.5–122°	121–123°	103–105°
Mixed melting point	119.5–121.5°			
Melting point of picrate	164.5–165°		196–198° (9)	221–222° (2)
Ultraviolet absorption maximum in 0.1 N HCl	232 mμ 295 mμ	232 mμ 295 mμ	228 mμ 303 mμ	232 mμ 295 mμ
ε (in 0.1 N HCl)	12250 5750		7000 7600	12300 5650
$A_{232/295}$	2.13	2.17	0.92	2.18
R_F	0.12–0.13	0.13–0.15	0.19	
Color with acid ferric chloride	Orange-red	Orange-red	No reaction	Orange-red
Color with ceric nitrate	Burgundy with fading	Burgundy with fading	No reaction	Burgundy with fading

to the biological product. Furthermore, the infrared spectra of both compounds were in excellent agreement (Fig. 4). Finally, synthetic 6-OHN proved to be metabolically active. Nicotine-grown resting cells and crude extracts oxidized synthetic 6-OHN in a manner which suggested that it was indeed an intermediate of nicotine degradation.

However, synthetic 6-OHN and its picrate derivative possessed melting points which differed significantly from those of the biological product and its picrate. The melting points of the free bases differed by some 19° whereas the melting points of the picrate derivatives differed by some 60° (Table II). Since the synthesis of 6-OHN from nicotine leads to the formation of a racemic mixture (2), the disparity of the melting points was ascribed to the comparison of a racemic mixture with a levorotatory enantiomorph and thus was not considered significant.

With use of the 40 to 60 fraction, the net oxygen uptake with the racemic, synthetic product as the substrate was one-half of that with the biological product. However the crude extract oxidized both materials with the same oxygen uptake (Table

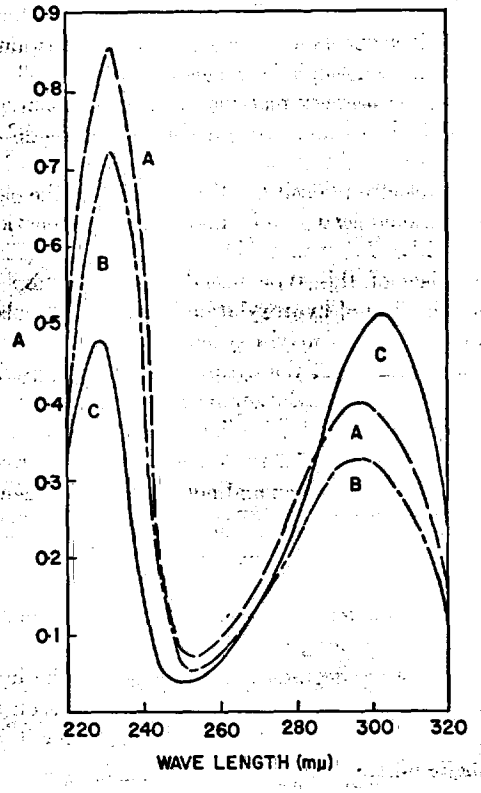

FIG. 3. The ultraviolet absorption spectra of the first oxidative product and synthetic 2-OHN and 6-OHN. *A*, first oxidative product (12.4 μg.); *B*, synthetic 6-OHN (10.4 μg.); *C*, synthetic 2-OHN (12.2 μg.). The absorption spectra was made in 0.1 N HCl.

myosmine. Preliminary evidence indicates that strain P-34 further degrades 6-OHN by a dehydrogenation of the pyrrolidine ring to yield 6-hydroxy-*N*-methylmyosmine, the methylated analogue of 6-hydroxymyosmine. Thus P-34, unlike the organisms studied by Wada, appears to degrade nicotine by a pathway similar to the pathway of nornicotine degradation suggested by Wada.

The degradation of nicotine by *Corynebacterium nicotinovorum* has been reported to yield *N*-methyl-2-(3-pyridyl)-1-pyrrollium hydroxide and *N*-methyl-2-(3-pyridyl)-1,2-pyrrollium hydroxide (11). The reported ultraviolet absorption spectrum of the former compound is in remarkable agreement with the spectrum exhibited by 6-OHN. The latter compound possesses an absorption spectrum essentially the same as the spectrum exhibited by our reaction mixtures after oxidation of nicotine to the 1 μmole of oxygen level. As the proposed products of *C. nicotinovorum* metabolism were not isolated in a state which permitted an unequivocal determination of their chemical and physical properties, no direct comparison with our product is possible. However, the marked similarity of the ultraviolet absorption spectra, although not conclusive evidence (12), would suggest that a reinvestigation of these compounds would be in order to determine whether nicotine degradation by *C. nicotinovorum* occurs by a pathway similar to that observed in strain P-34 or whether a different pathway of nicotine degradation does indeed exist.

By virtue of the requirement for methylene blue, the reactions leading to the formation of 6-OHN must involve a dehydrogenation step. As the reaction is accompanied by the consumption of 0.5 μmole of oxygen per μmole of nicotine, and the product contains 1 gm. atom of oxygen per mole, the product

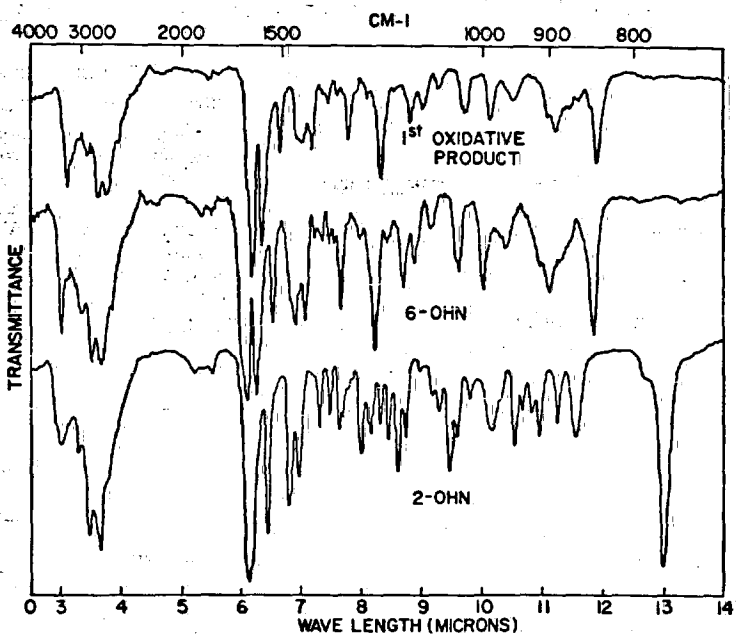

FIG. 4. The infrared spectra of the first oxidative product, synthetic 2-OHN, and synthetic 6-OHN.

nicotine, the demethylated analogue of nicotine. However, the metabolism of this compound was initiated by a hydroxylation reaction at the 6 position of the pyridine ring followed by a dehydrogenation of the pyrrolidine ring to yield 6-hydroxy-

oxygen must have its origin in water oxygen rather than molecular oxygen. Thus the over-all reaction can be conceived as being the sum of 2 reactions: one involving the addition of oxygen, the other the dehydrogenation step.

TABLE III

Oxidation of synthetic 6-OHN by cell-free extracts

Experimental conditions: 19.3 μmoles of synthetic 6-OHN, 12 μmoles of biological product, 50 μmoles of potassium phosphate buffer, pH 7, 0.5 ml. of crude extract, 4.2 mg. of 40 to 60 fraction. Total volume 2.0 ml., gas phase air 30°.

Extract	μMoles of oxygen per μmole of substrate	
	Synthetic 6-OHN	Biological product
Crude*	0.56	0.5
40 to 60†	0.27	0.5

* μMoles oxygen per μmole nicotine to the first rate change.

† μMoles oxygen per μmole of 6-OHN at the cessation of oxidation.

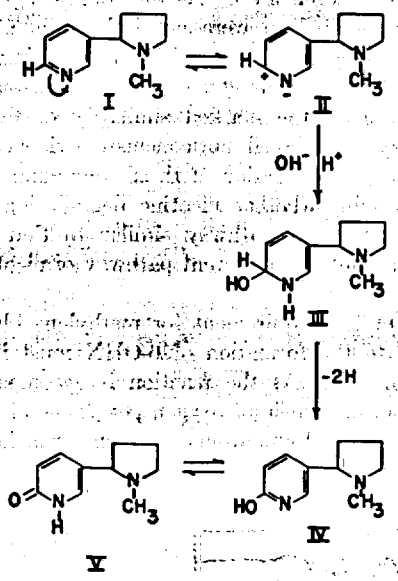

FIG. 5. The proposed pathway for the formation of 6-OHN from nicotine.

Since carbon-6 of the pyridine ring is a center of low electron density (13), it would be expected to be subject to nucleophilic attack. If the nucleophilic reagent is conceived as being hydroxyl ion, the primary reaction in the conversion of nicotine (*I*) to 6-OHN (*IV*) could proceed through a pseudobase (*III*) via a resonance hybrid of nicotine (*II*) in which carbon-6 is a center of low electron density. The addition of the elements of water to *II* would lead to *III* and a dehydrogenation of *III* would yield either *IV* or its pyridone tautomer (*V*) (Fig. 5).

A mechanism of this type would be a departure from the classical mechanism of hydroxylation in which molecular oxygen is the source of the hydroxyl oxygen found in the product (14). However, it may be that the hydroxylation of the pyridine ring is unique among hydroxylation reactions, for in the bacterial oxidation of nicotinic acid, leading to the formation of 6-hydroxynicotinic acid (15–17), the oxygen of the hydroxyl group has its origin in water oxygen and not molecular oxygen (15, 18).

SUMMARY

A compound, isolated from enzymatic reaction mixtures and from the growth medium, was shown to be the first oxidative product of nicotine degradation by a soil bacterium. This compound was identified as (1)-6-hydroxynicotine on the basis of its correspondence in properties, including infrared spectrum, with synthetic 6-hydroxynicotine.

A tentative reaction mechanism has been proposed in which the first step is the formation of a pseudobase by the addition of water across carbon-6 and the nitrogen of the pyridine moiety of nicotine, followed by the oxidation of the pseudo-base to (1)-6-hydroxynicotine, or its pyridone tautomer, by a methylene blue-dependent step.

Acknowledgment—The authors are grateful to Dr. Jerome A. Berson, Department of Chemistry, University of Southern California, for his advice in the field of heterocyclic chemistry.

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